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Genotoxic alterations in murine hepatocytes after short- and long-term exposure to N-nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, NNK, a component in tobacco cigarette smoke

A.M. Miyake-Mizusaka[1], T.L. Ikeda[1], M.K. Nagamine[1], R.P. Araldi[2] and M.L.Z. Dagli[1]

[1] Faculdade de Medicina Veterinária e Ciências Animais, Universidade de São Paulo, São Paulo, SP, Brasil
[2] Faculdade Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brasil

Corresponding author: M.L.Z. Dagli
E-mail: mlzdagli@usp.br

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ABSTRACT. There are over 7,000 components in cigarette smoke, 70 of which are considered genotoxic and carcinogenic. N-Nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is one of these components. When treated with NNK at concentrations of as low as 0.1 nM, breast and lung cells are known to acquire malignant properties. The liver plays an important role in toxin metabolization, yet little is known about the cytotoxic effects of NNK on hepatocytes. Therefore, we assessed the effects of NNK on immortalized murine hepatocytes (AML12 cell line) using a repeated exposure approach. AML12 cells were subjected to either short- (single exposure for up to 72 h) or long-term (cumulative exposure for 90 days) testing, at various NNK concentrations (0.1, 10, and 1000 nM). DNA damage was analyzed using the comet assay, a gold-standard technique to assess DNA strand breaks in eukaryotic cells. The cells subjected to short-term exposure had a significantly increased proliferation rate in the 0.1 and 10 nM groups when compared to controls. Furthermore, the cells from the 10 nM group exhibited increased migration rate after cumulative exposure to NNK. The clastogenic effect of NNK increased in a concentration-dependent manner up to 10 nM. We conclude that NNK is genotoxic and significantly alters cell viability and migration, contributing to malignant transformation of hepatocytes.

Key words: NNK; Hepatocytes; Smoking; Nitrosamine; Liver; Long-term exposure

INTRODUCTION

Tobacco use is associated with numerous chronic diseases, including several types of cancers (Ge et al., 2015; Cohen et al., 2018), and it accounts for more than eight million deaths each year globally (Pinto et al., 2015). In the USA alone, more than 16 million people were diagnosed with a disease caused by tobacco smoking in 2014. Tobacco smoking is one of the serious public health threats, especially in China, which has the largest number of tobacco users, including 288 million men and 13 million women (CDC, 2014; Asma et al., 2015; CDC, 2020).

Cigarette smoke contains over 7000 chemical components, of which 70 components have been identified as carcinogenic (IARC monographs, 2004; Rodgman and Perfetti, 2013). Nitrosamines, which are potent lung carcinogens (Hecht et al., 1980; Hecht et al., 1989), are components of cigarette smoke; they may induce carcinogenesis by DNA adducts and mutations (Xue et al., 2014). The most relevant tobacco-specific nitrosamine is N-nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Lung damage by NNK is well documented (Hecht, 1996; Hecht, 1999; Akopyan and Bonavida, 2006; Mennecier et al., 2014); however, it also affects other organs.

Repeated exposure to low concentrations of NNK can lead to the acquisition of cancerous properties by cells, such as anchorage-independent cell growth and invasive activity (Mei et al., 2003; Siriwardhana et al., 2008). Considering that the liver is an important organ for NNK metabolization, understanding the effects of NNK in hepatocytes is important; however, little is known about how it acts on such cells *in vitro*. Evaluating the *in vitro* effects of NNK on hepatocytes could help elucidate the action mechanism of NNK in the liver of individuals exposed to cigarette smoke. Additionally, *in vitro* toxicity studies are essential to help determine measures to control tobacco use and inform public health policies. Along this line, we examined the effects of short- and long-term exposure NNK on immortalized non-cancerous liver cell line AML12.

MATERIAL AND METHODS

Ethics

The study protocol was approved by the ethics committee of the University of Sao Paulo (Process Number 5026060317).

Cell culture and reagents

AML12 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). These cells, established by Wu et al. (1994), are non-tumorigenic murine hepatocytes derived from transgenic mice expressing human transforming growth factor alpha (TGF-α) (CD1 strain, line MT42).

The cells were cultured in Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM-F12; Gibco, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; Gibco), insulin-transferrin-selenium supplement (ITS-G; Gibco), 40 ng/mL dexamethasone (Sigma-Aldrich, USA), and antibiotic-antimycotic (Gibco) at 37°C in an incubator with a humidified atmosphere of 5% CO_2.

NNK was purchased from Toronto Research Chemicals Inc. (Ontario, Canada). A stock solution of NNK was prepared by dissolving 1 mM NNK in dimethyl sulfoxide (DMSO) and stored at -20°C until use. The stock solution was diluted in complete culture medium before each treatment.

Assessment of short-term exposure to different concentrations of NNK

According to the 45th series of workshops organized by the European Centre for the Validation of Alternative Methods (ECVAM), testing periods of up to 72 h are referred to as "short-term" (Pfaller et al., 2001). AML12 (0.3×10^4 cells/well) cells were grown in 96-well plates with 20% conditioned medium and 80% fresh growth medium. At 24 h post seeding, NNK was added at final concentrations of 0.1, 10, and 1000 nM; the cells were then incubated at 37ºC for 24, 48, and 72 h. The cells were subjected to a single exposure cycle of NNK.

MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted to assess cell viability (Mosmann, 1983; Van Meerloo et al., 2011). Three hours before the end of the cell incubation period, MTT (5 mg/mL) was added to each well and incubated in a light-protected area for the remaining incubation time. The microplate was centrifuged at 376 *g* for 10 min, and the supernatant was discarded; then, 100 µL of dimethyl sulphoxide (DMSO -Synth) was added to solubilize formazan crystals. The absorbance of the solution was read at 570 nm using a plate reading spectrophotometer (Varioskan, Carlsbad, CA, USA).

Assessment of long-term exposure to different concentrations of NNK

We adopted a repeated long-term exposure approach (Mei et al., 2003; Siriwardhana et al., 2008; Mennecier et al., 2014) to expose AML12 cells to three different concentrations of NNK (0.1, 10, and 1000 nM final concentration) for 90 days. The cells were seeded in culture flasks at 1×10^5 cells/flask. After 24 h, the medium was replaced with fresh complete medium containing NNK at final concentrations of 0.1, 10, and 1000 nM, and the cells were further incubated for 48 h. This 72-h exposure protocol was considered one cycle of exposure to NNK; it was repeated for a total of 30 cycles, spanning 90 days (Mennecier et al., 2014).

Wound healing assay

AML12 cells (control) and AML12 cells exposed to NNK for 30 cycles (0.1, 10, and 1000 nM) were seeded in 24-well plates (5×10^4 cells/well) and allowed to reach 100% confluence in culture media supplemented with 10% FBS. The cells were cultured under serum-free conditions for 24 h before the wound healing assay (Yue et al., 2010). The confluent cell monolayer was wounded using a 1-mL pipette tip, and then washed three times with phosphate-buffered saline (PBS) to remove floating cells. The scratch-wounded monolayer was cultured under serum-free conditions for 72 h. Untreated cells were also

cultivated in the presence of 10% FBS (assay positive control). Scratches were observed by microscopy (Zeiss, GE) and images were captured using the Image Pro Plus system at 0, 24, 48, and 72 h after wounding. All experiments were performed in quadruplicate.

The area of the lesion was evaluated using ImageJ (NIH.gov) to measure the migratory capacity of cells. With this approach, the wound closure percentage after scratching (time zero) can be calculated. The following equation was used to calculate the percentage of "wound" closure area (Yue et al., 2010):

$$\% \text{ "wound" closure} = [(A_{t=0\,h} - A_{t=\Delta\,h})/A_{t=0\,h}] \times 100 \quad \text{(Eq. 1)}$$

where, $A_{t=0\,h}$ is the area of the "wound" measured immediately after scratching, and $A_{t=\Delta\,h}$ is the area of the "wound" that was measured 24, 48, or 72 h after scratching.

Comet assay

Clastogenic potential of NNK at different concentrations was evaluated using the alkaline comet assay, owing to its high sensitivity to detect both simple and double-strand DNA breaks (SSDs and DSBs) (Collins, 2004). Slide preparation: glass slides (26 mm × 76 mm) were dipped in a solution of normal melting point agarose (NMA; Invitrogen, Carlsbad, CA, USA) diluted in PBS to 1.5% at 60°C, and one of the sides was cleaned with a paper towel. The slides were dried in a horizontal position overnight (Araldi et al., 2015; Araldi et al., 2018).

Sample preparation: The cells chronically exposed to NNK at concentrations of 0.1, 10, and 1000 nM were harvested, and the cell pellet was resuspended in 200 µL of PBS. Thereafter, 20 µL of cell suspension was added to 150 µL of low melting point agarose, and the solution (170 µL) was immediately transferred to the NMA pre-coated slides. The slides were covered with coverslips and maintained at 4°C for 20 min. The coverslips were gently removed, and the slides were placed in a Coplin jar containing lysis solution (2.5 mM NaCl, 100 mM EDTA, 10 mM Tris- HCl, 1.1% Triton X-100, and 11.2% DMSO) at 4°C, and incubated for one hour. This step was performed under dark conditions to prevent DNA damage.

Electrophoresis: After cell lysis, the slides were washed with PBS and transferred to an electrophoresis tank, filled with electrophoresis buffer of pH > 13.0 (300 mM NaOH and 1 mM EDTA) at 4°C for 40 min. Thereafter, electrophoretic run was performed with a current of 25V (0.86 V/cm), 300 mA for 20 min to promote the migration of free DNA fragments. The slides were transferred to a Coplin jar containing neutralizing buffer (400 mm Tris- HCl, pH 7.5) for 5 min. The material was fixed in absolute ethanol for 5 min.

The slides were stained with 20 µL of 20 µg/mL propidium iodide (PI) solution, and analyzed under epifluorescence microscope Carl Zeiss Axio Scope A1, equipped with an excitation filter of 510–560 nm and barrier of 590 nm, at 400× magnification, where 100 nucleoids were analyzed per slide, which were rated from 0 (without DNA damage) to 4 (maximum DNA damage). The scores were obtained by summing the product of the observed number of nucleoids per class with its respective class value.

Statistical analysis

Parametric data were assessed using an analysis of variance (ANOVA) followed by Bonferroni's post-hoc comparison test to compare the experimental groups. Non-parametric data were assessed using Kruskal–Wallis test followed by Dunnett's test for comparisons between groups. For statistical analyses, GraphPad Prism5.00® Software (GraphPad Software, Inc., San Diego, CA, USA) was used.

RESULTS

Short-term toxicity of different concentrations of NNK on murine hepatocytes

This assay was performed to evaluate the subacute cytotoxicity of various concentrations of NNK (0.1, 10, and 1000 nM) on AML12 cells after 24, 48, or 72 h of exposure. After 24 h, there was a significant increase in the viability of AML12 cells exposed to 0.1 and 10 nM NNK compared with control cells (Figure 1). This effect was more pronounced at 72 h after exposure in cells treated with 10 nM NNK.

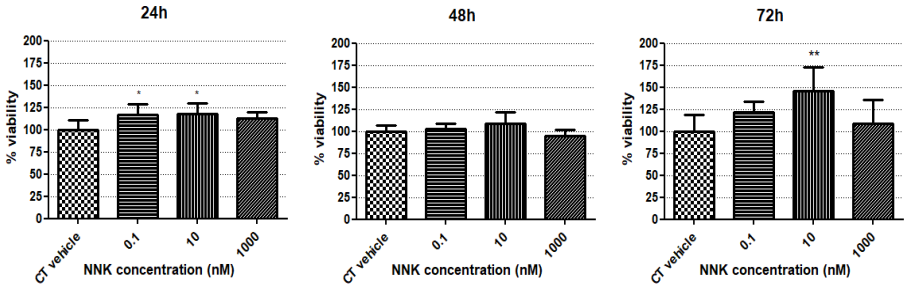

Figure 1. MTT assay. Viability of AML12 mouse hepatic cells after a single treatment with different concentrations of NNK. The cells were exposed to NNK in growth medium for 24, 48, and 72 h. ANOVA and Dunnett's post-hoc test. *P < 0.05; **P < 0.01.

Long-term toxicity of different concentrations of NNK on murine hepatocytes

Wound healing assay

Cells were grown until 100% confluence, and then a wound in the cell monolayer was created by scratching. Wounds were observed for 72 h under serum-free conditions. Scratch closure was documented by taking photographs at 0, 24, 48, and 72 h after treatment (Figures 2 and 3). AML12 cells exposed to 10 nM NNK for 30 cycles demonstrated a higher rate of cell migration, as observed by the decrease in the scratch area after 24 and 48 h, and almost complete wound closure after 72 h. This finding was similar to that of the positive control cells, which were cultured in growth medium supplemented with 10% FBS.

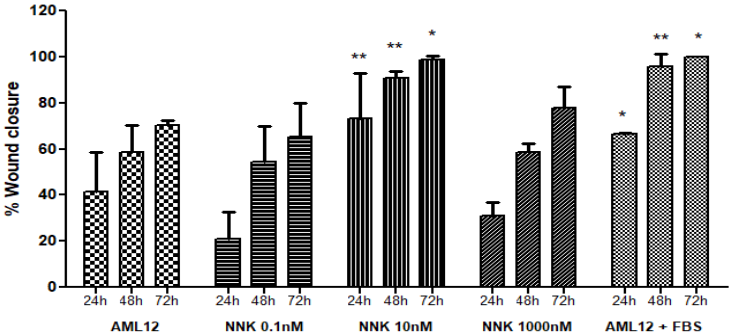

Figure 2. Wound healing assay. Bars show the percentage of wound closure at 0, 24, 48, and 72 h after scratching the cell monolayer. Cells exposed to 10 nM NNK for 30 cycles showed faster wound closure, similar to the assay positive control (AML12 cells cultured in medium with 10% FBS). The treatment groups were compared with AML12 cells (untreated group) at the indicated times. Statistical tests: Two-way ANOVA and Bonferroni. *P < 0.05; **P < 0.01.

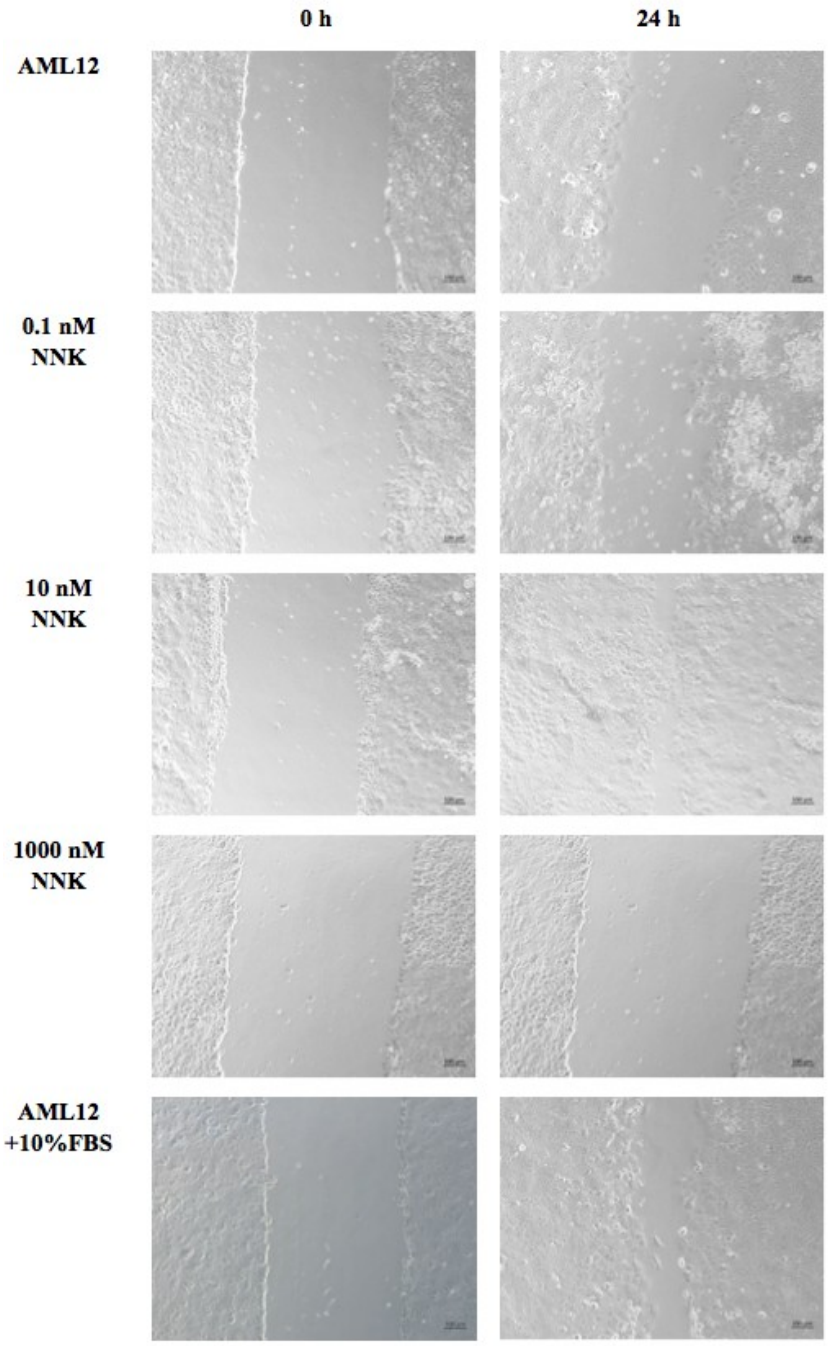

Figure 3. Photomicrographs of wound healing assay at 0 and 24 h after cell monolayer scratch. Untreated AML12 cells and AML12 cells exposed to NNK for 30 cycles were cultured in serum-free media for 72 h after scratching. Positive control cells were cultured in the presence of 10% FBS. Images were taken at 0, 24, 48, and 72 h to calculate the percentage of wound closure. Scale bar = 100 μm.

Comet assay

The comet assay was performed using AML12 cells that had been exposed to NNK for 30 cycles. One-hundred nucleoids per sample were counted and classified according to the degree of DNA damage, which was scored visually into three classes, from minimal damage (class 0) to maximum damage (class 2).

We observed a higher score (i.e., higher damage) in cells exposed to 10 nM NNK compared with that in non-exposed AML12 cells (Figure 4). This higher clastogenic effect was significant ($P < 0.05$, Kruskal–Wallis test). Interestingly, the highest concentration of NNK (1000 nM) resulted in less DNA damage than the intermediate concentration (10 nM).

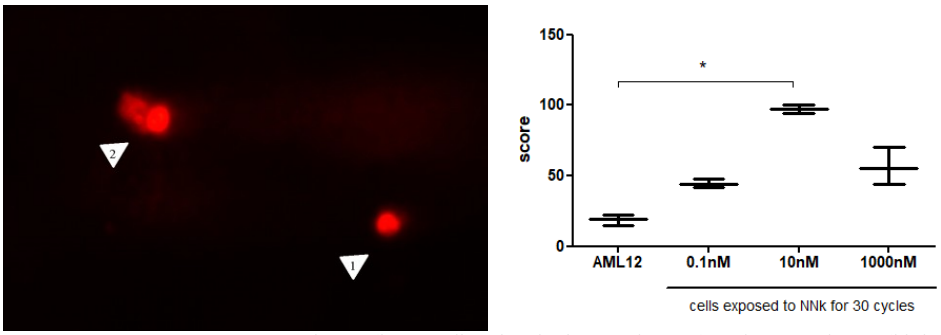

Figure 4. Comet Assay. A. Comet class 1 (intermediate level of DNA damage) and comet class 2 (high level of DNA damage). Original magnification was 40×. **B**. Comet scores showing differences in the levels of DNA damage in AML12 cells that were exposed to NNK at concentrations of 0 (AML12), 0.1, 10, and 1000 nM. Statistical tests: Kruskal–Wallis and Dunn tests. *$P < 0.05$.

DISCUSSION

Cigarette smoke is composed of various components as micro-particulates. NNK is one of the several carcinogens present in tobacco smoke and has strong carcinogenic properties (IARC MONOGRAPHS, 2004). Even at low concentrations, NNK exposure may result in the development of chronic diseases when directly or indirectly metabolized (Whitlatch and Schick, 2019). Considering that the liver is one of the main organs involved in drug and toxin metabolization, understanding how NNK can affect hepatocytes is important to control tobacco use and inform public health measures. The concentration of NNK that reaches the liver and the cytotoxicity of NNK for hepatocytes have not been fully elucidated *in vitro* and *in vivo*. It has been reported that NNK at a concentration of 0.1 nM can induce cancerous properties in breast (MCF10A) and lung cells (E10) (Siriwardana et al., 2008; Mennecier et al., 2014). Therefore, we used NNK at concentrations of 0.1, 10, and 1000 nM in our assays to represent cumulative exposure to tobacco. We focused on NNK as the study agent because the results may help elucidate the roles of NNK in the progression of liver cancer. In our study, we evaluated the effects of NNK after short- and long-term exposure on the murine AML12 cell lineage.

In the short-term testing, after a single exposure to different concentrations of NNK, we observed that cell viability was dependent on both drug concentration and exposure time. In the MTT assay, the 0.1 and 10 nM groups showed increased metabolism at 24 h of

exposure, when compared with the control, indirectly indicating a higher proliferation rate. This result was more pronounced in the 10 nM group after 72 h of exposure.

When investigating AML12 cells after 30 cycles of exposure to NNK, the group exposed at the concentration of 10 nM exhibited increased migration ability and DNA damage compared with the other groups. Cell migration is a part of tissue homeostasis and is also involved in pathological processes such as tumor invasion and metastasis (Yamaguchi and Condeelis, 2007). In our laboratory, the effects of long-term exposure to NNK at lower concentrations have also been demonstrated in E10 mouse lung alveolar epithelial cells. The results were similar to those of our study, resulting in increased migration, invasion, and proliferation rates, after cumulative exposure to NNK at a dose of 0.1 nM (Mennecier et al., 2014).

Interestingly, the 1000 nM group did not differ significantly from the control group with short-term and long-term exposures. We hypothesized that NNK has a biphasic dose response that has a stimulatory effect on cells exposed to NNK at low concentrations; as a result, NNK apparently exerts inhibitory or toxic effects when employed at higher concentrations. This type of response is known as hormesis, and it has important implications for mechanisms involved in carcinogenesis and its prevention (Mattson, 2007). Calabrese and Blain (2005) indicated that hormetic dose responses have been described in several biological models. In 2011, an updated analysis showed that nitrosamines are involved in 2% of the total cases of hormesis (Calabrese and Blain, 2011; Whitlatch and Schick, 2019).

Our findings demonstrated that NNK at concentrations of as low as 10 nM could stimulate cell metabolism and induce DNA damage. These effects are important when considering the concentration of toxins from cigarette smoke, which may reach the hepatocytes after cigarette smoking and that remain in the environment. A study analyzed the environmental persistence of third-hand smoke in a controlled chamber in Philip Morris Inc. between 1989 and 1992 and revealed that third-hand smoke, which is a mixture of tiny droplets and waxes from smoke, can be found in the environment for several months after a cigarette is extinguished (Whitlatch and Schick, 2019). The researchers of the study observed that NNK can be found in the air at a concentration of 1.5 pM in a non-ventilated room after a cigarette is smoked. They also demonstrated that NNK can persist on indoor surfaces such as carpets, curtains, wallpapers, and glasses, at even higher concentrations for more than 50 days.

NNK can create DNA bulky adducts (Peterson, 2016), whereby the carcinogen reacts with DNA to form covalent adducts. These adducts can clog the opening of DNA double strand and block or prevent the action of DNA polymerase (Xue et al., 2014) during DNA replication, thus leading to cumulative DNA damage. A study of *Caenorhabditis elegans* exposed to NNK at different concentrations reported damage to both nuclear and mitochondrial genomes (Bodhicharla et al., 2014). Owing to its high sensitivity, the comet assay is recognized as a gold-standard technique for quantifying DNA damage (Lacoste et al., 2006). Our study improves our understanding of the action mechanisms of one of the most important carcinogens in tobacco smoke, NNK, and its effects on hepatocytes. Our findings are also useful to inform and improve public health policies to protect and warn future generations against the harmful effects of tobacco use. Some aspects still require further elucidation; for example, further studies are necessary to clarify the biomolecular action of NNK in hepatocytes.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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